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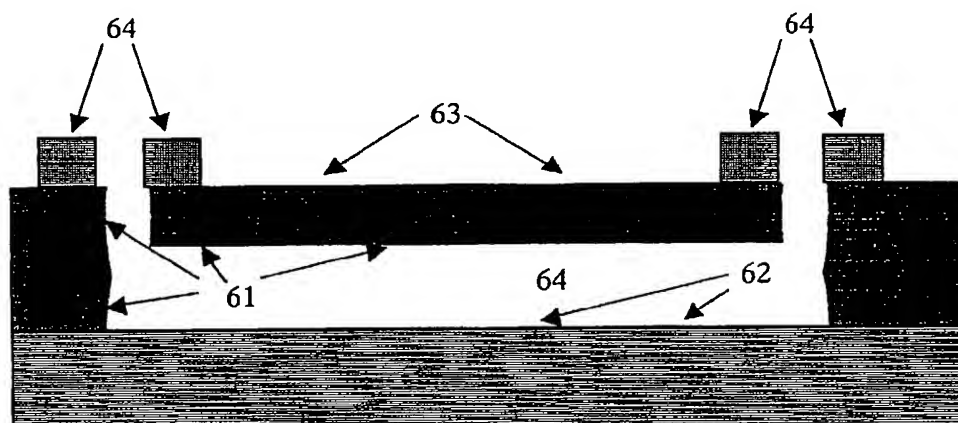
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LAYER BODY BY PLASMA ETCHING



(57) Abstract: The technology is based on the anisotropic plasma etching of organic polymer sheets partially protected by a metallic mask. The originality of the process is to pattern the surface properties by the same physical means as the one used for the three dimensional fabrication and simultaneously to this fabrication. Surface properties means, but are not limited to hydrophobicity, hydrophilicity, conductivity, reflectability, rugosity and more precisely the chemical and/or physical state of the surface. It is also possible to generate the desired functionalities, for instance carboxylic acid, ester, ether, amid or imid, during the etching process. The patterning of the different properties may be achieved by two different techniques that may be used separately or simultaneously.



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**METHOD FOR FABRICATING MICRO-STRUCTURES WITH
VARIOUS SURFACE PROPERTIES IN MULTILAYER BODY
BY PLASMA ETCHING**

FIELD OF THE INVENTION

The present invention provides a fabrication method for the three dimensional structuration and patterning of at least two different surface properties for micro-systems or micro-substrates.

5 BACKGROUND OF THE INVENTION

Over the last ten years, a general effort towards miniaturization of the analytical tools has been observed. Two main reasons are pushing the development miniaturized chemical apparatus, which have been called Micro Total Analysis Systems (μ -TAS): a decrease of analyte consumption and a decrease of duration of single analysis. Both
10 needs are particularly evident in the new development of life science, where genetic analysis and high throughput screening in drug discovery take more and more importance. In these applications, the reason for limiting the analyte consumption are evidenced by the increasing number of performed assays. In this case, the use of reactants for analysis must be as small as possible in order not only to reduce the

cost but also to limit the waste production. In other cases, the analysis of extremely small volumes is required. Such a volume may be only a few nL, e.g. in the case of neurological fluid analysis or in prenatal diagnostics. In many cases, the decrease in analysis time is also an important issue e.g. in medical diagnostics, where the time factor may signify a fatal issue for the patient. Two different and complementary strategies have been developed in parallel to achieve these goals. On one hand, the fabrication of microfluidic devices has allowed fluid handling in pL volumes and, on the other hand, immobilization of affinity reagents into high density 2-dimensional arrays for high throughput affinity analysis.

10 In recent years, capillary electrophoretic methods have enjoyed gaining popularity, primarily due to the observed high separation efficiencies, peak resolution, and wide dynamic ranges of molecular weights that may be analyzed. Furthermore, the simple open-tubular capillary design has lead itself to a variety of automation, injection and detection strategies developed previously for more conventional analytical technologies.

The general instrumental set-up involves a capillary filled with an electrolyte solution and a high voltage power supply connected to electrodes in contact with small fluid filled reservoirs at either end of the capillary. The power supply is operated in order to apply an electric potential field tangential to the capillary surface, in the range of 100-1000 V/cm. When the potential is applied, migration processes occur. The electric field imposes a force onto charged species leading to the electrophoretic migration of sample molecules within the capillary. Furthermore, when file capillary surface is charged, a flow of the whole solution is induced by electro-osmosis. Therefore, electrophoresis is in most cases superimposed on a so-called electroosmotic flow (EOF). Species moving in the capillary as a result of these forces will then be transported past a suitable detector, absorbance and fluorescence

being the most common. Capillary electrophoresis has been applied to numerous analytes spanning pharmaceutical, environmental and agricultural interests. A common focus amongst these activities is bioanalysis. Separation methods are developed for peptide sequencing, amino acids, isoelectric point determination for
5 proteins, enzyme activity, nucleic acid hybridization, drugs and metabolites in biological matrices and affinity techniques such as immunoassays. Furthermore, buffer additives such as cyclodextrins and micellar phases have added the ability to perform chiral separations of biologically active enantiomers of tryptophan derivatives, ergot alkaloids, epinephrines and others which is of great interest to the
10 pharmaceutical industry.

The capillaries described above generally have internal diameters between 50-200 μm and are formed in fused silica. The microfabrication of capillaries has also been accomplished by machining directly onto planar, silicon-based substrates. Silicon substrates have an abundance of charged silanol groups and thus generate
15 considerable EOF. In the case of micromachining, EOF can be an advantage in that the flow of the bulk solution can be used for many liquid handling operations. There has recently been intense activity in the area of chemical instrumentation miniaturization. Efforts have been made to reduce whole laboratory systems on to microchip substrates, and these systems have been termed micro-Total Analytical
20 Systems (μ -TAS). As already mentioned, most of such μ -TAS devices to date have been produced photolithographically on silicon-based substrates. This process involves the generation of the desired pattern on a mask, through which a photoresist coated silicon dioxide wafer is exposed to light. Solubilised photoresist is then removed and the resulting pattern anisotropically etched with hydrofluoric acid. Etched capillaries
25 are then generally sealed by thermal bonding with a glass cover. The bonding technique in particular is labour and technology intensive and thermal bonding requires temperatures between 600-1000 $^{\circ}\text{C}$. This bonding technique has a very low tolerance of defect or presence of dust and requires clean room conditions for the

fabrication, which means that the production is very expensive. Alternative fabrication techniques have also been developed based on organic polymers. Fabrication of polymer microfluidic devices has been shown by injection moulding or polymerising polydimethyl siloxane (PDMS) on a mould. These two techniques
5 have the advantage to replicate a large number of micro-structures with the same pattern given by the mould. Other techniques based on electromagnetic radiation either for polymerisation under X-ray (LIGA) or for ablation have also been recently shown to be feasible. This last fabrication technique allows fast prototyping by writing pattern on a substrate that can be moved in the X and Y directions. Different
10 structures can then be fabricated just by moving the substrate in front of the laser beam.

As already mentioned, electroosmotic pumping is used here not only to separate samples but also to dispense discrete amounts of reactives or to put in contact solutions for the reaction in continuous flow systems. A large diversity of structures
15 and electrical connections have been presented which permit to deliver and analyse samples in less than a millisecond by electrophoresis for example.

This spectacular property also evidences that, in these microchannels, the main transport mechanism between two flowing solutions is diffusion. As different species exhibit different diffusion coefficients, efficient mixing becomes problematic, and
20 this is often presented as a serious limitation for the wider use of microfluidic in total analysis systems. In order to solve this problem, mixers have been presented, where the flows are for instance divided in smaller channels (20 μm) before being placed in contact. In this manner, the diffusion time is reduced and hence the mixing efficiency enhanced.

Many recent advances in chemical analysis have involved the incorporation of biomolecules capable of selective and high affinity binding to analytes of interest. Such devices are often termed biosensors, which involve real-time transduction of the binding event into an electronic signal, but also include analytical technology consisting of immunoassays, enzyme reaction, as well as nucleic acid hybridisation. Bio-analytical devices utilising this technology have been applied to a wide range of applications in medicine agriculture, industrial hygiene, and environmental protection. Enzyme electrodes represent the oldest group of biosensors and are being increasingly used for clinical testing of metabolites such as glucose, lactate, urea, creatinine or bilirubin. Several groups have developed needle-type electrodes, for subcutaneous glucose measurements. A microelectrochemical enzyme transistor has been developed for measuring low concentrations of glucose. Efforts continued towards other clinically relevant metabolites particularly for the multiple-analyte determination. Strategy to incorporate affinity steps is also an active area of biosensors. The emerging area of DNA hybridisation biosensors has been a very popular topic for the clinical diagnosis of inherited diseases and for the rapid detection of infectious microorganisms.

Recent interest in the development of miniaturised, array-based multianalyte binding assay methods suggests that the ligand assay field is on the brink of a technological revolution. The studies in this area have centered largely on antibody or DNA spot arrays localised on microchips which are potentially capable of determining the amounts of hundreds of different analytes in a small sample (such as a single drop blood). Array-based immunoassay methods shows the particular importance in areas such as environmental monitoring where the concentrations of many different analytes in test samples are required to be simultaneously determined. Affymetrix developed ways to synthesise and assay biological molecules in a highly dense parallel format. Integration of two key technologies forms the cornerstone of the method. The first technology, light-directed combinatorial chemistry, enables the

synthesis of hundreds of thousands of discrete compounds at high resolution and precise locations on a substrate. The second laser confocal fluorescence scanning permits measurement of molecular interactions on the array.

5 Recently, the Laboratoire d'Electrochimie of the EPFL Lausanne has presented a patterning technique based on the photoablation process. In order to fabricate microarrays of proteins, the polymer substrate is firstly blocked with a bovine serum albumin (BSA) layer avoiding non specific adsorption of protein on the substrate layer. Microspots are then created on the surface by photoablation of the BSA layer, on which avidin can be adsorbed yet. This micropatterning technique allows then to
10 specifically adsorb antibodies linked to biotin on the avidin spots as visualised by biotin-fluorescein complex.

Apart from electrophoretic separations and hybridisation, an increasing number of applications on μ -TAS have been shown in the last few years. Full DNA analysers have been implemented in a single device with a polymerised chain reaction (PCR)
15 chamber followed by an electrophoretic separation. Continuous flow PCR has also been shown where the analyte solution is driven through a capillary crossing zones of different temperature. Other genetic analysis have also been demonstrated comprising high speed DNA sequencing, high density parallel separation or single DNA molecule detection. Another application of μ -TAS has been shown in
20 electrochromatography. An open-channel electrochromatography in combination with solvent programming has been demonstrated using a microchip device. Others have successfully used μ -TAS to conduct immunoassays involving competitive markers, noting several advantages over more traditional formats including (a) high efficiency separations between competitive markers and antibody-marker complexes,
25 (b) excellent detection limits (0.3-0.4 amol injected) at high speed, and (c) good potential for automation. This has first been demonstrated in a micromachined

capillary electrophoresis device by Koutny et al. Cortisol was determined in serum using a competitive immunoassay that was subsequently quantitated using μ -TAS. A microfluidic system was fabricated on a glass chip to study mobilisation of biological cells on-chip. Electroosmotic and/or electrophoretic pumping were used to drive the cell transport within a network of capillary channels. An automated enzyme assay was performed within a microfabricated channel network. Precise concentrations of substrate, enzyme and inhibitor were mixed in nanoliter volumes using electrokinetic flow. Finally, the new insight in the use microfabricated system has been to combine the advantage of parallel reactions and liquid handling in extremely small volumes with an electrospray or nanospray interface for mass spectrometry analysis. This last application opens a way to efficiently use the microchip format not only for genetic analysis where it is already recognised but also in protein sequencing.

Several microfabrication processes have been shown that modify the surface properties of the polymer.

It is known that reactions of gas plasmas with polymers can be classified as follows:

1. Surface reactions:

Reactions between the gas-phase species and surface species produce functional groups and/or crosslinking sites at the surface.

2. Plasma polymerisation:

The formation of a thin film on the surface of a polymer via polymerisation of an organic monomer such as CH_4 , C_2H_6 , C_2F_4 and C_3F_8 in a plasma.

3. Cleaning and etching:

Materials are removed from a polymer surface by chemical reactions and physical etching at the surface to form volatile product.

Patent of particular relevance in the etching process:

5 US 5099299 (Dyconex)

Patent with particular relevance in lamination sealing of polymer micro-structure:

WO 991197 17 (Aclara Biosciences)

Patent of particular relevance in patterning of properties:

WO 9823957 A (EPFL)

10 Other patents on microfabrication and fluidic control by surface properties:

WO 9823957 A (EPFL)

WO 9846439 (Caliper technology)

WO 9807019 (Gamera Bioscience)

SUMMARY OF THE INVENTION

According to the present invention, an etching method for manufacturing micro-structures or openings in a multilayer body made of selected materials is provided, wherein the surface properties of the layers are controlled individually and preferably
5 simultaneously control, so that conduits, grooves, reservoirs, holes and so forth are formed and exhibit various surface properties allowing further functionalisation of selected surfaces as well as fluid handling.

Further, an etching method of the above kind is provided where the conduits and
10 reservoirs are manufactured in sequential etching steps between which selected layers can be removed or added to the multilayer body or between which the properties of selected etched surfaces can be modified.

Still further, such an etching method is provided for manufacturing micro-structures or openings in a multilayer body which can contain a selected sequence of insulating
15 and optically or electrically conductive layers, whereby measurement and/or detection of one or more analytes and/or fluid handling means are provided.

Also, such an etching method is provided where a plurality of layers are manufactured simultaneously or where etching processes are used.

Thus, the present invention provides a fabrication method for the three dimensional structuration and patterning of at least two different surface properties for micro-systems.

5 The invention further comprises products manufactured using this method as defined in the claims.

The technology is based on the anisotropic plasma etching of organic polymer sheets partially protected by a metallic mask. The originality of the process is to pattern the surface properties by the same physical means as the one used for the three dimensional fabrication and simultaneously to this fabrication. Surface properties
10 mean, but are not limited to hydrophobicity hydrophilicity, conductivity, reflectability, rugosity and more precisely the chemical and/or physical state of the surface. It is also possible to generate the desired functionalities, for instance carboxylic acid, ester, ether, amid or imid, during the etching process. The patterning of the different properties may be achieved by two different techniques that may be
15 used separately or simultaneously.

1. The fabrication of multilayer of polymer of different properties, for example, a sandwich composed of two thin layers of electrical insulator (polystyrene) spin coated on both sides of a conducting polymer sheet (carbon filled polystyrene). The plasma etching cuts vertically the three layers, letting
20 appearing a band of conducting band isolated by two others.
2. The substrate partially protected by a mask on both sides is placed in the middle of two chambers (A and B), separated hermetically, inside which a plasma is generated differently in chamber A than in chamber B. For this,

the surface exposed to chamber A is treated with an oxidative plasma (O_2) while the other one with a non-oxidative plasma (N_2). The surface of such a hole would be half hydrophilic and half hydrophobic with respect to the etching rate of both plasma.

- 5 In a preferred embodiment, the technology may be applied to manufacture micro-analytical systems that are devoted to many applications, like for instance chemical and biological analysis, synthesis and/or separation. Furthermore, in another embodiment, the technology may serve to manufacture devices devoted to reactions occurring at the interface between a liquid and a solid surface or at the interface
10 between two solutions.

For example, microelectrodes or micro-needles may be fabricated and used for electrochemical detection or in mass spectrometry sampling. The system may be used for liquid extraction between two phases like partitioning experiments. Furthermore, the technology may be applied to every kind of induced flow like
15 diffusion, convection (for example by electroosmosis) or migration (for example by electrophoresis). The technology may also be used for applications where the plasma created surface is chemically or biochemically derivated in order to perform chemical or biochemical assays. As further example, the technology may be applied to reaction types where the temperature may be adjusted and/or controlled for
20 instance by the use of electrical means like integrated thermistors or thermocouples, as for example for PCR reactions.

BRIEF DESCRIPTION OF THE DRAWINGS

- Fig. 1A-1E show schematic sectional views through a portion of an embodiment of the multilayer body showing methods for manufacturing micro-structures or openings in this multilayer body which is coated on both sides and which is made of a plurality of materials,
- 5 Fig. 2A-2E show schematic sectional views, through a portion of an embodiment of the multilayer body showing methods for manufacturing micro-structures or openings in this multilayer body which is coated on both sides and which is made of a plurality of materials, one of the layers already containing micro-structures or openings,
- 10 Fig. 3A-3C show schematic sectional views through a portion of an embodiment of the multilayer body showing methods for manufacturing micro-structures or openings in this multilayer body made of a plurality of materials using a plurality of etching processes allowing to process one layer and to remove another one simultaneously,
- 15 Fig. 4A-4C show schematic sectional views through a portion of an embodiment of the multilayer body showing methods for manufacturing micro-structures or openings in a multilayer body made of a plurality of materials, using a plurality of etching processes allowing to discriminate the structuration of two different portions of a layer,
- 20 Fig. 5 is a schematic diagram showing a method for processing a plastic film of indeterminate length in accordance with the present invention,

Fig. 6A-6E schematically show a sequence of microfabrication with plasma etching,

Figs. 7A-7C show a side view of micro-structures fabricated by plasma etching with the electrodes and the lamination,

5 Fig. 8 is a top view of the unsealed micro-structure, together with closer views of the electrode microdisk inserted in the microchannel,

Fig. 9A shows the voltammetric detection of ferrocene carboxylic acid in the microchannel in three electrode mode versus Ag/AgCl (ferrocene carboxylic acid concentration from 0 to 500 μM in 125 mM PBS pH 7.4 + KCl 100 mM),
10

Fig. 9B represents the ferrocene carboxylic acid concentration versus the current at 400 mV vs. Ag|AgCl,

Fig. 10 shows the voltammetric detection of glucose at 15 mM in the microchannel in three electrode mode versus Ag/AgCl (ferrocene carboxylic acid concentration 100 μM in 125 mM PBS pH 7.4 + KCl 100 mM),
15

Fig. 11A shows the voltammetric detection of different concentrations of glucose in the microchannel in three electrode mode versus Ag/AgCl

(ferrocene carboxylic acid concentration 100 μ M in 125 mM PBS pH 7.4 + KCl 100 mM),

Fig. 11B represents the glucose concentration versus the current at 400 mV vs Ag|AgCl inside the microchannel,

5 Fig. 12A shows the voltammetric detection of different concentrations of glucose on the pads in three electrode mode versus Ag/AgCl (ferrocene carboxylic acid concentration 100 μ M in 125 mM PBS pH 7.4 + KCl 100 mM),

10 Fig. 12B represents the glucose concentration versus the current at 400 mV vs Ag|AgCl on the electrode pads,

Fig. 13 shows the configuration used here for the electrokinetic pumping at 1100 volts and the simultaneous electrochemical detection. This structure is a top view of the structure presented as a cross section in Figure 7c, and

15 Fig. 14 shows the electrochemical detection of ferrocene carboxylic acid pumped by electroosmotic flow in the device of Figure 13 (1 mM of ferrocene carboxylic acid in 10 mM phosphate buffer at pH 7.4).

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The term "micro-structure", as used herein, means and refers to a single micro-channel, an array of micro-channels or a network of interconnected micro-channels not limited in shape but having a cross-section enabling micro-fluidic manipulations. In accordance to the present invention, these "micro-structures" are usually formed in
5 e.g. a plate, a planar substrate or the like, and they are usually made in at least two layers, one containing the desired micro-structure pattern and a second one serving as sealing component.

The term "openings", as used herein, means and refers to hollow passages or spaces. These openings include for example reaction chambers, reservoirs, wells and the
10 like. They can stand alone or can be positioned at either end of a channel. When such openings stand alone, they can for instance be used for reagent introduction, mixing, incubation, washing, reaction, detection and the like. as required in e.g. homogeneous assays. When connected to a channel, they are for instance used as means for introducing a fluid into a main channel or a channel network. When going
15 through a plurality of layers, these openings can also be used to form a micro-structure having selected portions of various surface properties.

In the present invention, "channels" and "micro-channels" are conduits or means of communication (e.g. fluid communication) between openings and the like. They include for instance trenches, grooves, flumes, capillaries and so forth, without
20 limitation in shape. The "micro-channels" are yet limited to 0.1 - 1000 μ m in at least one of their dimensions.

The "surface properties", as this term is used herein, mean and refer to the chemical and/or physical state of the surface. They for instance include hydrophobicity, hydrophilicity, conductivity, reflectability, rugosity, sieving, affinity and so forth.
25 The term "conductivity" refers here to the ability of a surface to transfer electrons

from another material or solution into its bulk or, in the opposite, to transfer electrons from its bulk to another material or solution in contact. Those surface properties are intrinsically related to the nature of the materials used to form each layer, and, in accordance with the present invention, they can be modified in some parts of a multilayer body during the structuration process. In some embodiments, the surface properties of selected parts of a multilayer body can be further modified after the structuration process. The surface properties for instance serve to control the displacement or not of a medium within the formed micro-structures or openings. In accordance with the present invention, the surface properties can be selected in various parts of a multilayer body in order to, for instance, prevent or favour capillary flow, electroflow (i.e. electrokinetic flow, electroosmotic flow, electrophoretic flow, dielectrophoretic flow and so forth) chromatographic retention, molecule binding (e.g. adsorption or physisorption), optical or electrical conductivity, and so forth.

Fig. 1 to 4 show different manners of manufacturing micro-structures in a multilayer body with simultaneous control of the properties of the etched surfaces. In some embodiments, the multilayer body is a plastic film having an etch resist coated on one or both sides. The term "etch resist" refers herein to a substance which is resistant to the etching medium or, at least, is much more resistant than the material to be etched.

In a preferred embodiment, plasma etching, i.e. a technique in which the etching medium is gaseous, is used preferably to other techniques such as wet chemical etching or photoablation due to the difficulty of the former to provide the necessary precision required to manufacture micro-structures and due to relatively low processing speed of the latter. It is yet possible to use combinations of these methods

in order to further modify selected surfaces of etched layers in order to modify their functionality.

The precision of the plasma etching method directly depends upon the precision of the pattern structured in the etch resist coatings and upon the thickness of the layer to be etched. Any available methods like, for instance, the photochemical processes used in the electronics industry can be used to structure the etch resist like, for instance, a photoresist with micrometer precision. Plasma etching has the further advantage to allow for a directional etching (anisotropic plasma etching), which prevents lateral etching of material below the etch resist, a phenomenon called "underetching". Furthermore, the etch resist can be removed after micro-structure or openings fabrication when the material of the etch resist is not desired. This is for instance the case of plastic films that have been metallised e.g. by vacuum metal deposition before the etching process, but that cannot be constituted of a metal for their applications. Finally, the main advantage of plasma etching with respect to the present invention is that the etching medium can be varied in order to pattern the desired surface property of selected materials.

Fig. 1 to 4 show different manners of micro-structuring polymer layers providing various surface properties to the etched surface of each material composing the multilayer body. The figures are not to scale and represents only a portion of the entire bodies. They also present different stages of an etching process taking place from both sides of the multilayer body, even though each side of the multilayer body can be processed sequentially.

Fig. 1A shows a portion of a multilayer body for instance made of a plastic film 3 sealed on both sides with a laminate 2, 2' made of a second material that is coated by an etch resist 1, 1'. The central plastic film is, for example, 100 μm thick

polyethylene terephthalate (PET), whereas the laminate film is 25 μm thick polyethylene (PE) sealed to the first layer by any available technique. The etch resist can be a metal such as copper with a thickness of 12 μm which has been applied by a known electrolytic process, by laminating, by sputtering or any other available
5 technique. This etch resist already contains recesses such as 4 and 4' that have the shape necessary to manufacture the desired pattern, and that are located at the desired positions where openings are to be formed. The preliminary steps of photoresist application on both sides of the body and further development of this photoresist coating to obtain the recesses 4 and 4' of the desired pattern are not presented in any
10 of the below figures, their fabrication being not an object of the present invention.

In Fig. 1B and 1C, openings 5, 5' and 7, 7' are etched successively through the layers 2, 2' and, respectively, 3, thereby resulting in passages exhibiting different surface properties 6, 6' and 8. In Fig. 1D, the etch resist 1 is removed by any available method, as may be required for various applications. Similarly, the etch resist can be
15 coated with another layer (not shown) for instance for interfacial connections of the metal coatings. Any of these etching steps can be preceded by a treatment in a solution, not shown, for reducing the etching time. Furthermore, any of these etching steps can be followed by a treatment in order to modify the surface properties of the structured openings. In the example where the body is a PET film sealed to a copper
20 coated laminate PE film, the surface of the PET film is made highly hydrophilic during an oxidative etching process (as with oxygen plasma etching), whereas the surface of the PE remains much less hydrophilic. In this case, a drop of aqueous solution deposited on the copper coating 1 will not be able to enter the opening 7 by capillary fill. An external force must be applied to this drop to let it reach the
25 hydrophilic surface 8. Once the Surface 8 is in contact with the drop, capillary fill is induced in this portion of the micro-structure, but it is stopped as soon as the solution front reaches the second hydrophobic surface 6'. Here again, an external force is necessary to let the fluid front penetrate into the opening 7'. This example illustrates

one manner of handling fluids in micro-structures formed according to the present invention. Etching providing surfaces of medium hydrophobicity can also be used to slow down the fluid flux in it given portion of a micro-structure, which can be advantageous to complete a reaction, an adsorption and so forth in the case where
5 longer times are needed.

In Fig. 1E, the structured multilayer body is coated by a supplementary layer 9 using any conventional method, such as for instance lamination, in order to seal one end of the formed structure, thereby providing a micro-structure with an opening only at the opposite end.

10 Fig. 2 shows different stages of a fabrication process totally similar to that clarified for Fig. 1. The only difference consists in the fact that the central layer 3 contains one or more micro-structures or openings 10 located at the desired position(s) either to prevent (not shown) or to allow connection with the opening to be etched. In this last case, the shape of the complete micro-structure formed by the etching process is
15 modified, as well as the extent of the surface properties 8 patterned during this etching process. In another variation, the micro-structure(s) or opening(s) 10 is (are) made of a third material such as e.g. a polymer, a gel, a paste and so forth or is (are) filled with an assembly of materials such as fibers, waveguides, beads and so forth.

Fig. 3 and 4 show two different ways of fabricating micro-structures in different
20 layers using a plurality of etching processes. In Fig. 3, layer 11 is resistant to a first etching process and contains the recess 15 to produce the desired pattern in layer 12. A second etching process is then used to fabricate the desired micro-structures or openings to simultaneously remove layer in layer 13, without affecting the surface properties 16 of the previously etched layer and creating different surface properties
25 17 in layer 13. In the present case, the layer 11 only serves as an etch resist for the

first fabrication step, because it is not desired for the use of the structured body. If this layer is prejudicial to the second etching process, it can be removed before structuring layer 13. In another embodiment, layer 11 can be selected in such a manner that it is resistant to the first etching process, but not to the second, so that both layers 11 and the desired pattern in layer 13 are etched simultaneously. In Fig. 3, the etched micro-structures or openings do not extend through layer 14 which is resistant to both etching processes. However, this is not a necessity of the process, and the multi layer body can be selected in such a manner that both sides can be etched simultaneously following the above procedure. Furthermore, the above operations can also be repeated several times in order to fabricate micro-structures and openings in a body containing a larger number of layers.

Fig. 4 shows a method similar to that presented in Fig. 3 for the structuring a multilayer body and the patterning of surface properties of various natures in different layers. The etch resist 15 contains a plurality of recesses 20 and 21, and the second layer is made of a plurality of materials (two materials 16 and 17 in the case shown). None of the etching processes is able to attack the etch resist 15, and this layer is not removed between two fabrication steps. Materials 16 and 17 are selected in such a manner that only material 16 is resistant to the first etching process, so that a recess is created in layer 17 only. In a second step, a second etching process is used to produce the desired micro-structures of openings either in layer 16 only, either in layer 18 only (cases not shown) or in both layers 16 and 18 simultaneously. This leads to a three dimensional structure where holes 20 and 21 have different surface properties depending on the nature of the layers and on the step during which they are etched. In the present example, surfaces 22, 23 and 24 can have different properties or, if layers 16 and 18 are of made of similar materials, surfaces 23 and 24 have the same properties whereas 22 is different.

It must also be stressed that the surfaces of the etched micro-structures described in any of Fig. 1 to 4 can be further treated to bind, immobilise or coat a molecule in selected materials and/or selected layers. This can for instance be applied to immobilise biological molecules on a portion of a layer in order to perform a separation or an assay. Affinity chromatography, enzyme linked immuno-sorbent assays, receptor binding assays are some examples of the applications of the micro-structures manufactured according to the invention. Similarly organic material as for instance lysine, polyacrylamide or sodium dodecyl sulfate can be attached to selected etched layers in order to perform electrophoresis.

Fig. 5 shows a continuous process for producing micro-structures and openings in plastic films. A supply roll 31 supports the multilayer body 32 that can be either coated with an etch resist on one or both sides containing preformed recesses or not. Small rolls 33 direct the multilayer body through various process stages and steps 40 to 43, and the final end of the multilayer body is wound up on a take-up roll 39 to collect the final product. This step-up can for instance be used to process the structure shown in Fig. 4. A first stage 40 comprises all the steps required to coat the multilayer body 32 with an etch resist 15 containing recesses 20 and 21. In a metal etching and photoresist shipping stage 41 etching of metal coatings 15 takes place at the location of recess 20 where the micro-structures and/or openings are to be formed. In the next process stage 42, another second process is used to etch layers 16 and 18 simultaneously, thereby creating the desired surface properties 22, 23 and 24 in each material. During the last process stage 43, the etch resist 15 is removed, and the structured multilayer body is finally sealed by laminating a supplementary plastic film 38, yielding the final product 39.

Further process stages can also be added to the strip installation, and the various process stages can be devoted to other functions like washing, curing, coating,

surface modification, immobilisation, and so forth. Similarly, layers can be added to the body between two or several process stages. This is illustrated in Fig. 5 by the supplementary roll 34 that allows to laminate a plastic film 35 that is for instance used as a sealing of the etched micro-structures and openings formed in previous process stages and/or as a supplementary etch resist for the next process stages.

In the following, an example of an experiment carried out using the method according to the invention and its results is described in order to exemplify the concept of the invention.

For the experiment, polyimide foils coated on both sides with 5 μm thick copper are used as substrate material.

In a first step, plasma etched micro-structures are fabricated. Plasma is a highly excited state of matter, typically that of a diluted gas, in which a certain percentage of the gas atoms and molecules are ionised and then split to form highly reactive gas radicals. These chemically aggressive particles react preferentially with organic materials and generate reaction by-products which are subsequently desorbed from the surface. If the surface of an organic dielectric is partially covered with a metal mask, only the open areas can be attacked.

40x40 cm^2 polyimide foils of 50 μm thickness and coated on both side with 5 μm copper are fixed in a frame. The copper is chemically etched after patterning of photoresist with the help of a computer printer, e.g. a 25'000 dpi high resolution printer.

In **Figures 6A-6E**, the manufacturing sequence for a double-sided foil with plasma-drilled micro-structures is shown schematically. Figure 6A shows a foil 50 coated on both sides by a copper layer 51. In Fig. 6B, these copper layers are then covered by a photoresist 52 which is further exposed to light in such a manner that two holes 53 and 53' and one recess 54 are created, as shown in Fig 6C. This multilayer body is then etched chemically in order to structure the copper layers and create holes 55 and 55' and recess 56 of the same patterns as those made within the photoresist layers (Fig. 6D). The polymer foil is then structured by exposition to plasma in order to create an inlet 57 and an outlet 57' reservoir on one side and a groove 58 on the other side (Fig. 6E).

Due to the fact that plasma has access to the substrate from both sides, the holes 57 and 57' and the groove 58 are formed simultaneously when the copper 51 has been patterned on both sides of the foil 50. After this process, the surface state of the polymer can be very hydrophobic or hydrophilic depending on the plasma composition that is either O₂, CF₄ or N₂. In the below examples, oxygen plasma has been used in order to get an oxidised surface that can generate capillary flow inside the microchannels. Nevertheless, the surface outside of the capillary, protected by the copper layer will remain hydrophobic.

The above process can be repeated in order to create structured portions of different level (various depths), thereby producing recesses, cavities, protruding features and the like. This can for instance be used to create contact among the various layers constituting the multilayer body.

In another example, this process is used to integrate electrodes within the device. To achieve this, well-defined portions of the structured device (as for instance portions of the groove 58 shown in Fig. 6E) are exposed again to the plasma through a novel

copper mask containing the desired patterns. In this manner, the polymer foil 50 can be further etched until the copper layer 51 is reached. After these steps, a metal such as for instance gold is electroplated on the copper layer in order to get a surface which is suitable for electrochemistry purposes.

- 5 An example of such a plasma etched device is shown in **Figure 7A**. In the present case, the device is produced in a 50 μm thick polyimide foil, and it contains: one micro-channel 58 with one inlet 57 and one outlet 57', as well as two micro-electrodes 60 that are gold coated copper pads. The final structure is then sealed by lamination of a 35 μm thick polyethyleneterephthalate-polyethylene (PET-PE) layer
10 60 (Morane LTD, UK) with the same procedure as the one already presented elsewhere.

It is very important to observe the surface properties of the channel after the fabrication process, which is schematically described in **Figure 7B**. Indeed, inside the microchannel, the surface 61 is charged and hence hydrophilic, which is
15 necessary to enable capillary and/or electroosmotic flow. The wall of the sealed micro-channel made of the laminated layer 61 is yet less hydrophilic due to the nature of PE. Outside of the capillary, the surface 63 must be hydrophobic, so as to avoid the dispersion of the drop of solution around the openings serving as inlet and outlet. In the below examples, polyimide, which is an hydrophobic material, is
20 chosen for that purpose, since it becomes hydrophilic upon exposition to the oxygen plasma. Another surface property is the conductivity of the surface 64 where the metallic layer is in contact with the solution. These structures therefore demonstrate the concept of the invention: patterning different surface properties that are needed for controlling of the fluid flows, performing chemical reactions, detecting analytes and
25 so forth.

Figure 7C shows another example of distribution of the above surface properties, where electrodes are placed directly above the inlet and outlet of a sealed micro-channel.

Next, the electrochemical detection is performed by cyclic voltammetry with an
5 AEW2 portable potentiostat (Sycopel Scientific, UK) by connecting one of the electrodes as working electrode (WE) and another one as counter electrode (CE). A freshly oxidised Ag|AgCl wire is used as reference electrode and placed on the top of one channel entrance in contact with the solution to be analysed. Cyclic voltammetry characterisation of ferrocene carboxylic acid is first presented to understand the
10 behaviour of the gold coated microelectrodes similarly to what was presented earlier in a previous paper.

Now, micro-structures fabricated according to the present invention are then used to demonstrate some examples of analytical applications, namely immunological assays and enzymatic reactions.

15 For the example of immunoassay, the immobilisation of the mouse antibodies was performed by physisorption at pH 7 during one hour at room temperature. Depending on the experiments, between 1 and 100 µg/ml of antibody concentration is used. The surface is then blocked with 5% Bovine Serum Albumin (BSA). The immunoreaction is performed by filling dried channels with immobilised mouse
20 antibody with a goat antimouse-HRP conjugate and incubating it 5 minutes at dilutions between 1/225'000 and 1/25'000 titre. After the incubation with the conjugate, the substrate solution containing 100 mM Hydroquinone and 100 mM peroxyde is added to the channel to allow the electrochemical detection of Horse-

Raddish-Peroxidase (HRP) with a similar procedure as that proposed by Wang et al. Between each step, a washing procedure is performed with a solution of washing buffer at pH 7.4 and containg 0.1 M phosphate buffer and 0.1 % BSA.

5 In a second example, the use of the microchip is demonstrated for an enzymatic assay. Plasma etched micro-structures have been used here for the detection of glucose. A solution of Glucose oxidase (enzyme) and ferrocene carboxylic acid (mediator) is mixed with a solution of glucose and filled in the microchannel where a cyclic voltammetric detection is performed.

10 In another schema, the Glucose oxidase and ferrocene solution is filled into the micro-channel, and the glucose solution is placed on one of the reservoirs.

In a further example, 2 μ L of glucose oxidase and ferrocene carboxylic acid is deposited and let dried on the electrode pads outside of the channel. Then a solution of glucose is deposited on the dried solution and a cyclic voltammetry is performed.

In the following, the obtained results are shortly described.

15 First, the aspect of the structures used is addressed. Microscopy examination of the plasma etched plastic foils 70 before sealing by lamination of a PET-PE layer shows the different patterns that compose the micro-structure. Four top views of the device are presented in **Figure 8**, which contains a yellow-brown colour due to the light absorption of the polyimide layer 70. In the upper view on the left, the presence of
20 the micro-channel 71 is shown as a hell pattern in the middle of the image, meaning that the thickness of polyimide at this place is smaller. At both ends of the channel,

there is a hole 72 that serves as reservoir or as inlet and outlet, thereby allowing to access the liquid inside the channel after the lamination procedure. The other lines patterned on the surface 73 are the gold coated pads to connect the electrodes with the potentiostat. In the closer views of the device presented in **Figure 8**, it can be observed that the geometry of the electrode is a disk that is slightly recessed from the channel level.

It is worth noting that the upper view on the left side of **Figure 8** also shows series of four holes 74 that are used for the precise alignment of the device during its fabrication process.

10 Electrochemical characterisation: The cyclic voltammetric analyses of ferrocene carboxylic acid presented in **Figure 9** exhibits an expected shape for microelectrodes of these dimensions in a microchannel.

A calibration of ferrocene carboxylic acid can be obtained between 0 and 0.5 mM with a slope of 34 pA/ μ M, which is about 6 times larger than what was obtained in a similar geometry with a 5 times smaller carbon band electrode. The performance of these electrodes are in good agreement with such earlier work and can be used for diagnostics assays.

Glucose detection with plasma etched microchips: In a first experiment, the reaction is performed by mixing the enzyme and the mediator solution with a 15 mM glucose solution in a test tube outside the microchip. This solution is then injected in the microchannel and a cyclic voltammetry experiment is performed. The detection of glucose in such microchip can be shown in **Figure 10**. Without the presence of glucose in the solution, the voltammogram shows the oxidation of ferrocene

carboxylic acid as in Figure 9. The presence of glucose is revealed by the catalytic shape of the voltammogram, meaning that the mediator is reduced and oxidised by the enzyme and the electrode respectively. This shows that the detection of glucose is possible within this microchannel. It is worth adding that the volume of the micro-channel is about 50 nL in this example.

In a second experiment, the glucose oxidase and ferrocene carboxylic acid solution is filled in the microchannel. Solutions of different concentrations of glucose are then deposited on the reservoir at the outlet of the microchannel. The glucose is finally detected by cyclic voltammetry as presented in Figure 11A. The current detected at 400 mV is also plotted in Figure 11B against the glucose concentration. A good correlation of the glucose concentration and the detected current is evidenced between 0 and 20 mM. For larger glucose concentrations, the detection reaches a saturation.

In a third experiment, 2 μ L glucose oxidase and ferrocene carboxylic acid is dried on the electrode pads outside of the microchannel. In this experiment, 2 μ L solution of glucose is added on the electrode pads and the recorded voltammograms are presented in Figure 12A. The correlation of the current versus the concentration (Figure 12B) is linear from 0 to 20 mM. It is interesting to compare the current intensities between the detection inside the microchannel (Figures 10 and 11) and on the electrode pads outside of the microchannel. The current is larger in this last experiment because of the difference in the electrode dimension. The volume of detection in this last case is 2 μ L versus 50 nL inside the microchannel.

Another example of application is now shown to demonstrate that the present invention can be used to manufacture micro-structures in which the walls are

hydrophilic enough to generate a capillary flow and to control the movement of the fluids by electrical means. To this aim, the device schematically presented in **Figure 13** has been produced in a 50 μm thick polyimide foil 75 following an etching process similar to that described in Figure 6. The device of Figure 13 contains a 10
5 cm long micro-channel 76 with one inlet 77 and one outlet 78 at each extremity. These inlet and outlet also serve as reservoirs, and they are surrounded by two gold coated copper pads 79 and 80 that are used as electrodes. In the outlet reservoir, a platinum electrode 81 and a silver/silverchloride (Ag/AgCl) reference electrode 82 are put in contact with the solution. A high electric field (1100 Volt) is then applied
10 between electrodes 79 and 81, so as to electrokinetically pump the solution through the micro-channel 76 towards the outlet 78. The arrow in Figure 13 shows the direction of the flow generated by the application of this high voltage. A low potential (for example 400 mV vs Ag/AgCl) can also be applied between electrodes 80 and 82 in order to detect the molecules reaching the outlet reservoir.

15 Preliminary experiments showed that it is possible to aspirate solution through such microchannels in order to fill and empty them easily. Further experiments have then been undertaken for the characterisation of the electroosmotic flow generated in sealed microchannels of the shape shown in Figure 13.

To this aim, a solution of ferrocene carboxylic acid (1 mM of ferrocene carboxylic acid in 10 mM phosphate buffer at pH 7.4) is placed at the inlet of the microchannel and pumped in the direction of the low voltage detection set-up placed at the outlet. As soon as the pumped electroactive species reach the outlet of the microchannel, a current is detected by the electrochemical system defined by electrodes 80 and 82. As shown in **Figure 14**, when the solution only contains the phosphate buffer, the
25 current remains close to zero. A current is only detected at the beginning of the experiment, which is an artefact due to the switching of the potential. When the

ferrocene carboxylic acid solution is added at the inlet of the micro-channel, the current remains the same as that recorded for the phosphate buffer during 150 seconds. After these 150 seconds, the current rapidly increases until it reaches a plateau after approximately 200 seconds. This clearly shows that the ferrocene
5 carboxylic acid has been electrokinetically pumped through the micro-channel, and that it needed approximately 150 seconds to reach the outlet reservoir.

This experiment demonstrates that it is possible to use electroosmotic flow in micro-systems produced by the present invention and hence to use them to perform electrophoretic separations as a chromatographic technique.

- 10 The three experiments shown here evidence the great interest of using the present structure or kind of structures fabricated by plasma etching for applications in chemical or biological analysis.

Enzyme linked immunosorbent assay (ELISA) with electrochemical detection: In order to develop an immuno-diagnostic assay, antibodies can be immobilised on the
15 surface of the channel walls. The procedure is performed on the basis of physisorption or by covalent attachment. Then, standard immunoassay in sandwich or competitive mode can be performed. The detection can be achieved for example by having labeled the secondary antibody or the antigen with an enzyme such as but not limited to HRP, ALP, glucose oxydase, beta-galactosidase, etc. Structures and
20 arrays or networks of structures similar to those shown in Figures 6 to 8 can then be used for such immunoassays, since appropriate surface properties can be patterned using the present invention.

Nanospray fabrication: The structure fabricated and presented in Figure 7 can be used for mass spectrometry analysis. Indeed, if the structure are cut either with a knife, a laser or by plasma, the cross section of the channel can be placed in front of a mass spectrometer inlet, and the high voltage required to spray the solution out of the capillary can be applied thank to the electrode fabricated inside the capillary. The interior of the channel (that is hydrophilic) serves to let the channel be filled and the outlet of the channel (that is hydrophobic) serves to favor the fabrication of the Taylor cone. Indeed, the exterior must be hydrophobic to prevent the aqueous solution to spread outside of the channel, thereby favoring the generation of the spray.

These examples demonstrate the use of the present invention even if it is not limited to these applications.

WHAT IS CLAIMED IS:

1. An etching method for manufacturing micro-structures and/or openings in a multilayer body made of a plurality of materials, in which surface properties of said materials are controlled individually by the etching process, said
5 micro-structures or openings and said surface properties being created simultaneously.
2. A method according to claim 1 wherein at least one micro-structure or at least one opening is manufactured through the entire thickness of at least one of said layers.
- 10 3. A method according to any of claims 1 and 2 wherein said body contains means for assembling said layers in precise relative positions for desired alignment of said micro-structures and openings.
4. A method according to any preceding claim wherein at least one micro-structure in one of said layers has at least one dimension in the range 0.1 to
15 1000 μm .
5. A method according to any preceding claim wherein each of said layers has a maximum thickness of 1 cm but is not limited in length, width and shape.

6. A method according to any preceding claim wherein said layers exhibit either hydrophilic or hydrophobic surface properties depending on said material used.
- 5 7. A method according to any preceding claim wherein at least one portion of said micro-structures is filled with a medium, said medium being either a fluid, a solid or a gel.
8. A method according to claim 7 wherein said medium is a fluid containing charged particles, such as ions, molecules, cells or viruses.
- 10 9. A method according to any preceding claim wherein at least one layer of said body is made of a plurality of materials
10. A method according to any preceding claim wherein at least one material in said multilayer body is an optically conductive material, an electrically conductive material, an organic material, a metal, a polymer or glass.
- 15 11. A method according to claim 10 wherein said electrically conductive material serves as electrode.
12. A method according to claim 11 wherein said electrode is emplaced and is provided with leads tot, connection to a source of electrical power.

13. A method according to claims 11 and 12 wherein said electrically conductive material is used to induce a displacement of said medium and/or of said charged particles in at least one portion of said micro-structures.
14. A method according to claim 13 wherein said charged particles are separated
5 and/or mixed during said displacement.
15. A method according to any preceding claim wherein at least two different layers are etched simultaneously.
16. A method according to claim 15 wherein said micro-structures are manufactured from both sides of said multilayer body.
- 10 17. A method according to any preceding claim wherein manufacturing said micro-structures or openings in said multilayer body comprises plasma etching, photoablation, or chemical etching.
18. A method according to claim 17 wherein said micro-structures or openings in
15 said multilayer body are etched using a plurality of etching processes or under a plurality of atmosphere including but not limited to either air, oxygen, nitrogen, hydrogen, argon or fluor.
19. A method according to any preceding claim wherein at least one supplementary layer is added to said multilayer body after etching.

20. A method according to claim 19 wherein said supplementary layer already contains one or several said micro-structures or openings.
21. A method according to any preceding claim wherein at least one layer is removed from said multilayer body after etching.
- 5 22. A method according to any preceding claim wherein the surface properties of at least one layer is further modified by at least one physical or chemical treatment.
- 10 23. A method according to claim 22 wherein molecules are immobilised on at least one portion of said layer by either physisorption, chemisorption, covalent binding or ionic binding.
24. A method according to claim 22 wherein said further modification of said surface properties results from the deposition of a metal.
25. A method according to claim 22 wherein said further modification of said surface properties results from a polymerisation reaction.
- 15 26. A method according to any preceding claim wherein said micro-structures are manufactured in sequential fabrication steps.

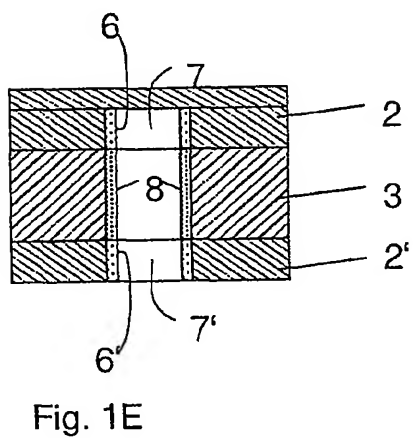
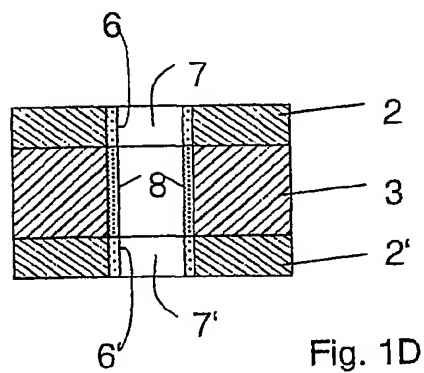
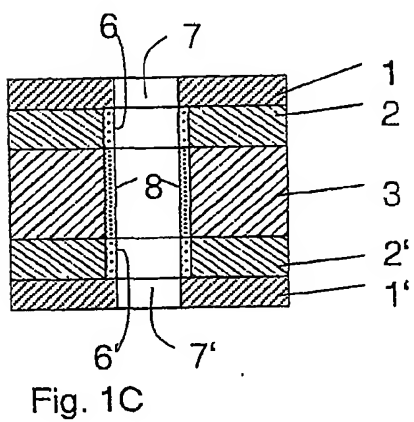
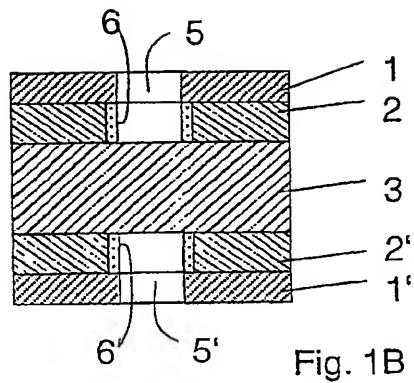
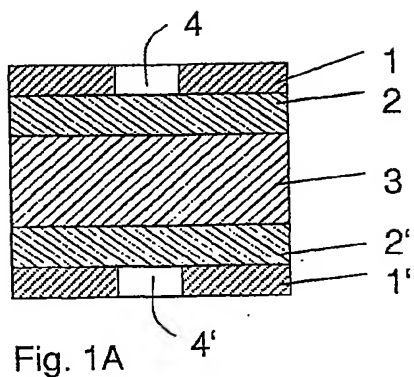
27. A method according to claim 26 wherein at least one layer is added to said multilayer body after at least one said fabrication step.
28. A method according to claim 27 wherein one fabrication step includes derivatisation and/or immobilisation of material on the etched micro-structure or microstructures by pouring the multi layer body into a solution containing the desired reagent for such derivatisation and/or immobilisation.
29. A method for manufacturing interconnected and /or interconnectable hollow and/or solid micro-structures in a multilayer body made by an etching process; in which the layer material of the micro-structures are individually chosen for some or for all of the layers or in which the etching process is chosen such that desired bulk or surface properties are produced or maintained during the etching process and/or in which some surface properties are realized by a following step of activation of the surface of some or all of the micro-structures; so that the multilayer body embodies interconnected and/or interconnectable micro-structures of partially different physical or chemical surface properties or activities respectively.
30. Multilayer body comprising a substrate layer and at least two added layers and micro-structures formed in or by at least said two added layers, said two added layers having different chemical and/or physical surface properties so that the micro-structures have sections with differing surface properties, whereby transport, analysis or treatment of fluids, i.e. of liquids or gases, in said micro-structures is enabled.

31. Multilayer body comprising a substrate layer and at least one added layer and micro-structures formed in or by at least said substrate layer and said added layer, the surface of said substrate layer further being conditioned to have surface properties different from the surface properties of said added layer so that the micro-structures have sections with differing surface properties, whereby transport, analysis or treatment of fluids in said micro-structures is enabled.
32. Multilayer body comprising at least two connected substrate layers wherein at least one of said two substrate layers comprises a surface pointing to the other one of said two substrate layers with micro-structures formed thereon so that at least a microchannel is formed between said two substrate layers, the microchannel comprising a surface with sections having differing chemical and/or physical surface properties, whereby transport, analysis or treatment of fluids in said microchannel is enabled.
33. Multilayer body according to claim 32, comprising two substrate layers adjacent to each other, wherein at least one of said two layers comprises a groove formed in the surface adjacent to the other one of said two layers, so that a microchannel is formed.
34. Multilayer body according to any one of claims 30 to 33 comprising a network of premanufactured interconnected and/or interconnectable hollow and/or solid micro-structures of partially different physical or chemical surface properties.

35. Multilayer body according to any one of claims 30 to 34, wherein at least one said micro-structure or opening comprises a surface section being hydrophobic and a surface section being hydrophilic.
- 5 36. Multilayer body according to claims 32 and 35, wherein the layers comprise an electrically insulating foil forming a first substrate layer and a lamination forming a second substrate layer and further comprising a microchannel arranged between said insulating foil and said lamination, the surface between said microchannel and said insulating foil being charged and other surface sections of said insulating foil not being charged.
- 10 37. Multilayer body according to any one of claims 30 to 36, wherein surface sections of at least one of said micro-structures have different conductivities, reflectabilities, rugosities, sieving rates and/or a different corrugation.
- 15 38. Multilayer body according to any one of claims 30 to 37, wherein surface sections of at least one of said micro-structures have different physisorption and or different chemisorption rates of a particular material.
39. Use of the multilayer body according to any one of claims 30 to 38 to perform chemical and/or biological analysis, e.g. chemical or biological assays, such as immunoassays or enzyme assays.
40. Use of the multilayer body according to any one of to claims 30 to 39 for

electrical and/or mechanical induced flow practices.

41. Use of the multilayer body according to any one of claims 30 to 40 for separation techniques, such as electrophoresis, chromatography and so forth.
42. Use of the multilayer body according to any one of to claims 30 to 41 as a
5 sensor and/or an electrospray tip.
43. Use of the multilayer body according to any one of to claims 30 to 42, wherein said multilayer body is coupled to an analytical system like for instance a liquid chromatograph, a capillary electrophoresis apparatus, an isoelectric focusing system, a size discrimination device and so forth.



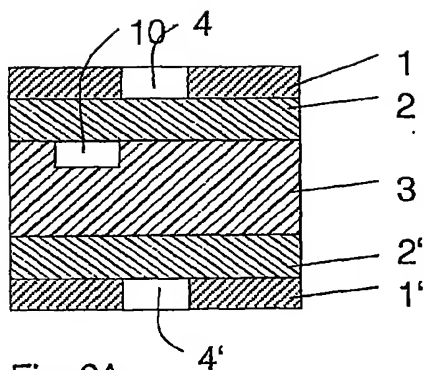


Fig. 2A

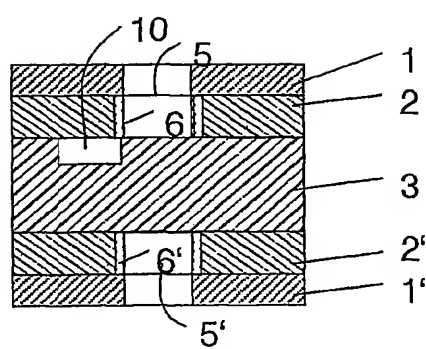


Fig. 2B

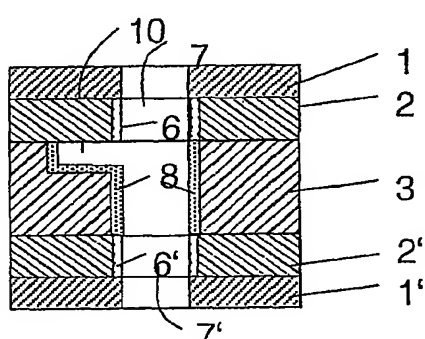


Fig. 2C

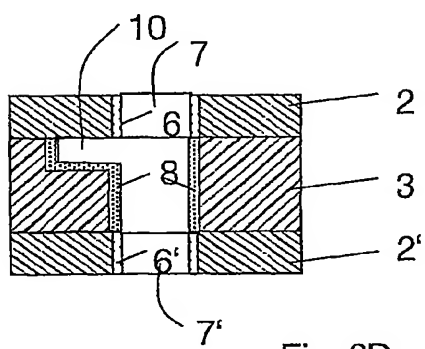


Fig. 2D

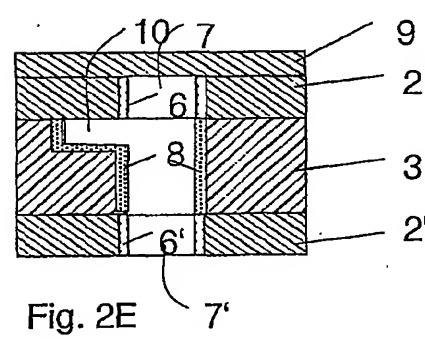


Fig. 2E

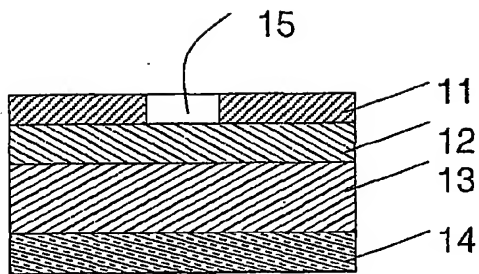


Fig. 3A

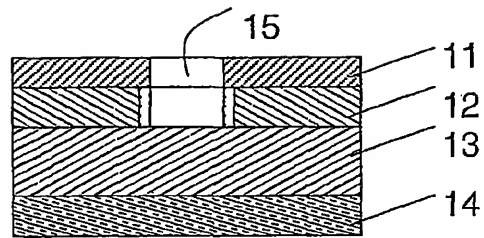


Fig. 3B

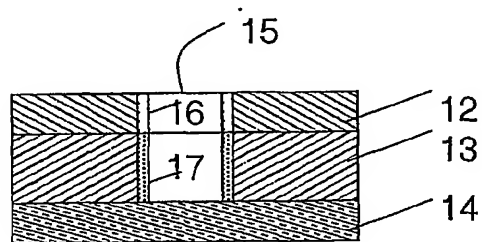


Fig. 3C

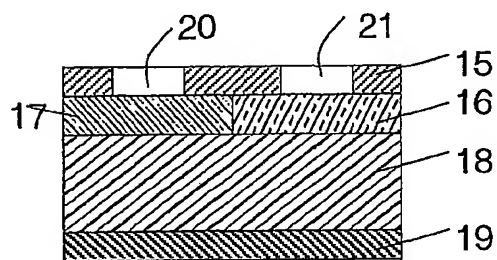


Fig. 4A

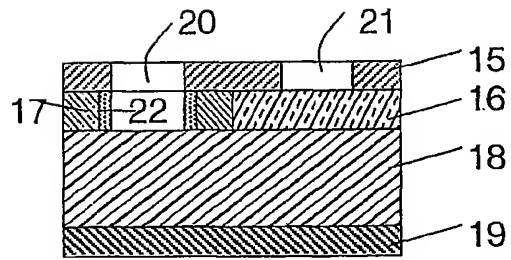


Fig. 4B

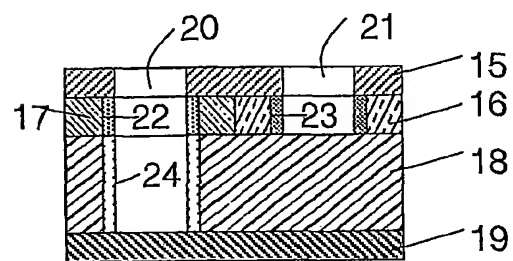


Fig. 4C

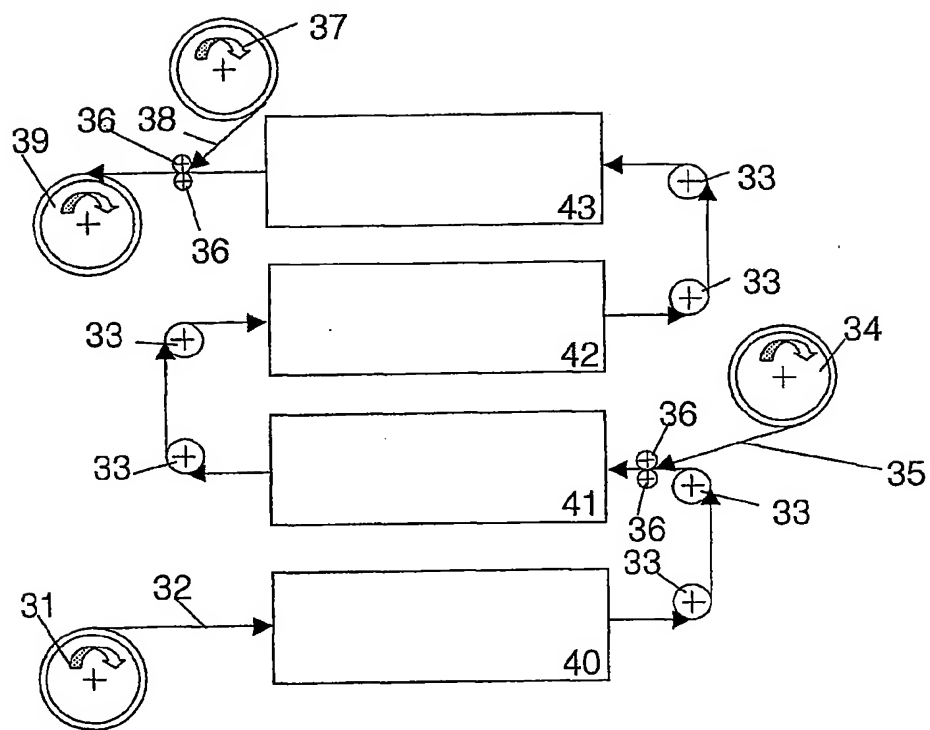


Fig. 5

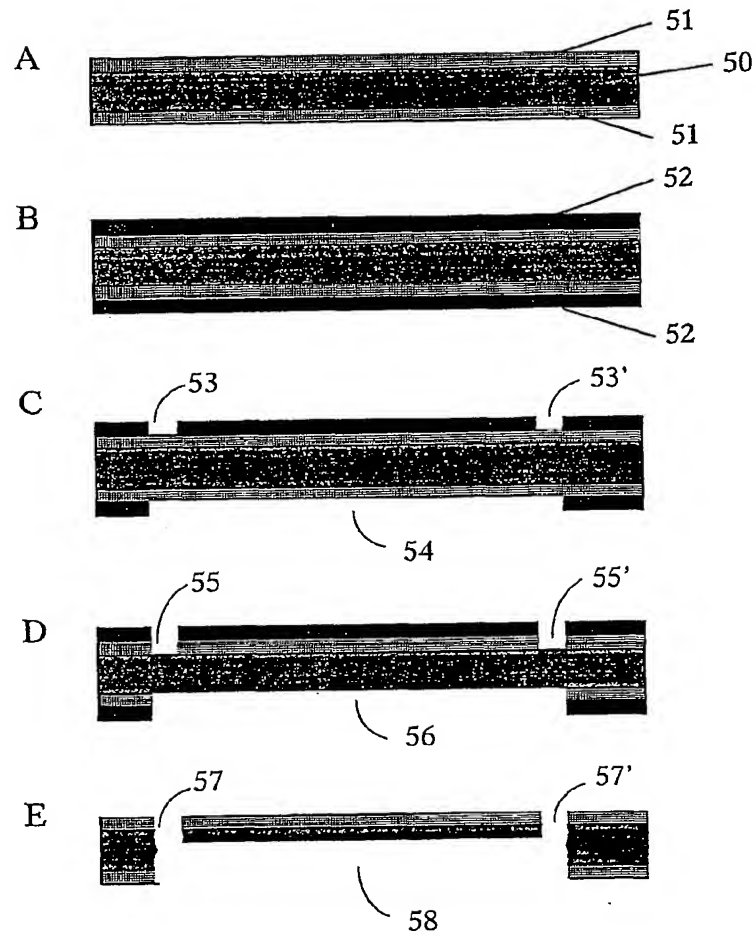


Fig 6

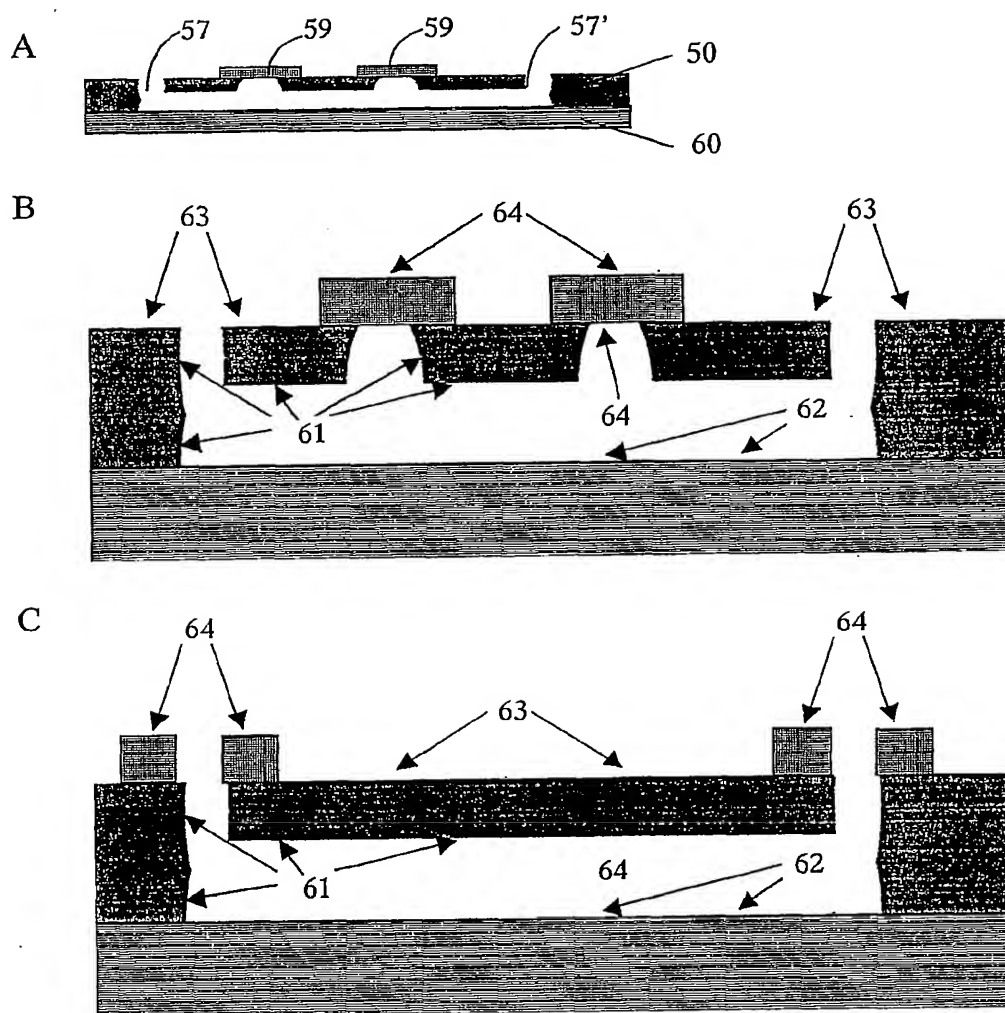


Fig. 7

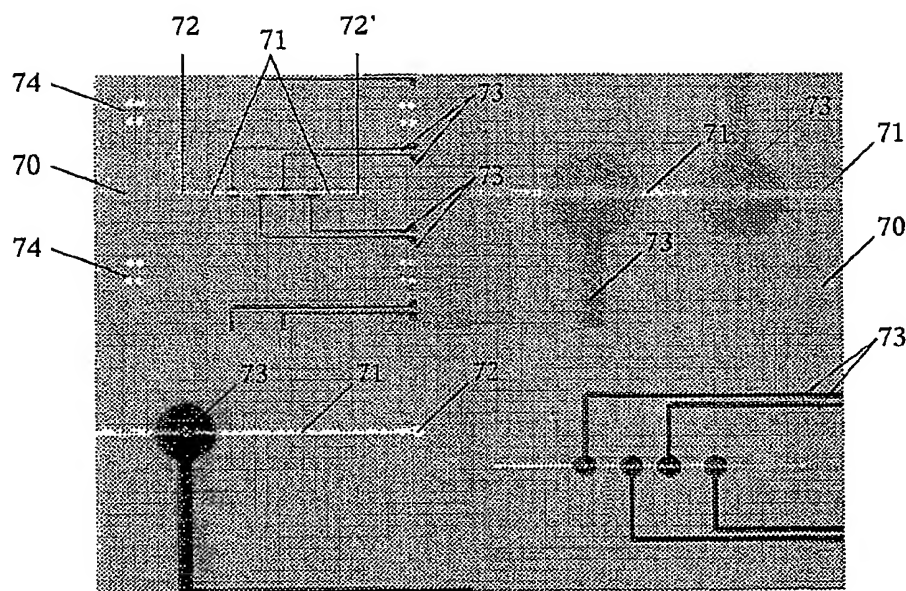


Fig. 8

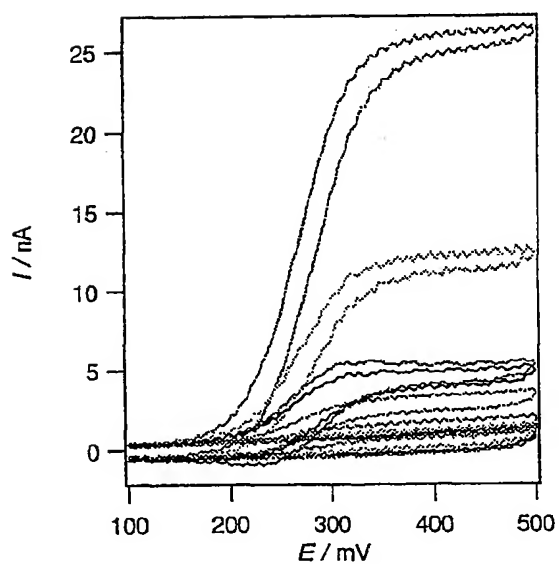


Fig. 9A

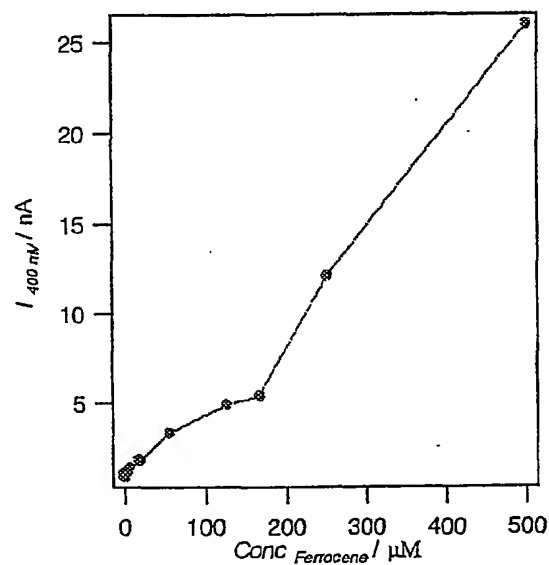


Fig. 9B

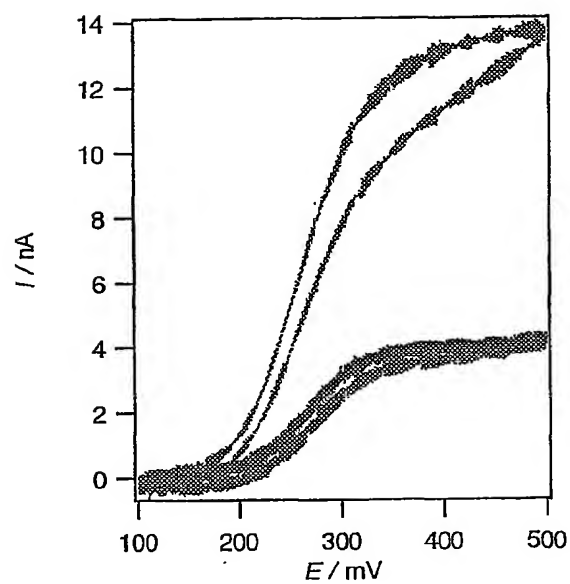


Fig. 10

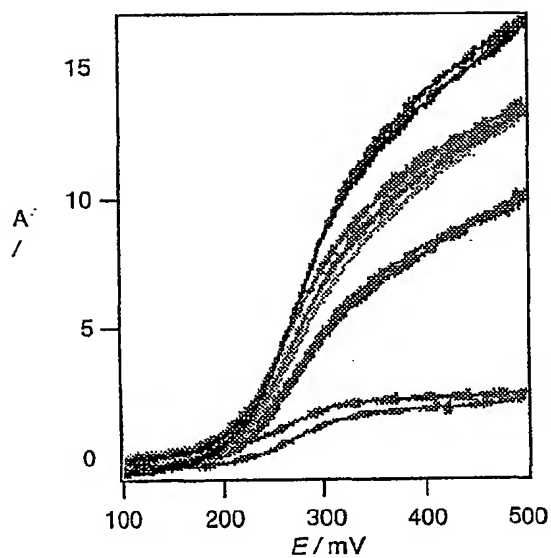


Fig. 11A

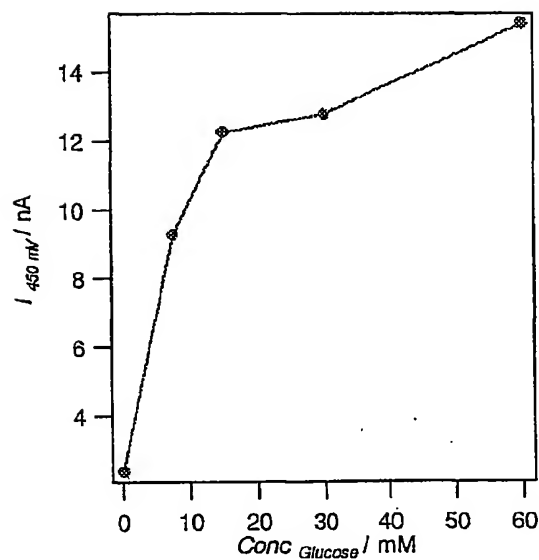


Fig 11B

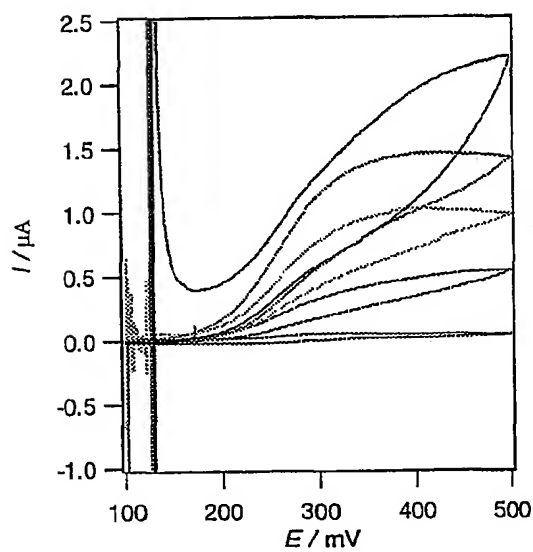


Fig. 12A

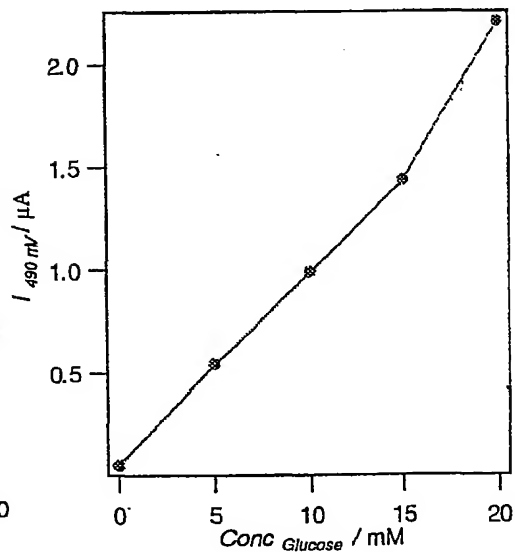


Fig 12B

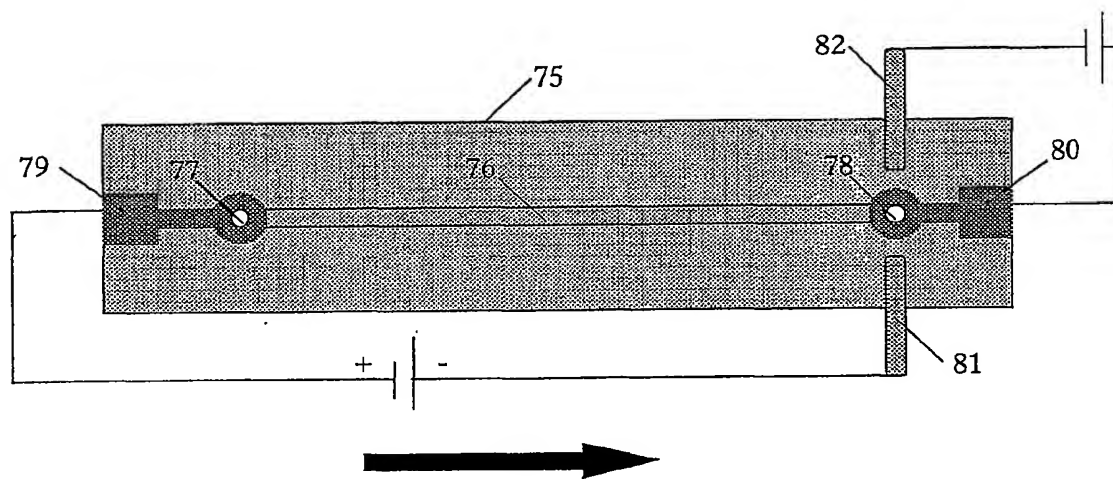


Fig. 13

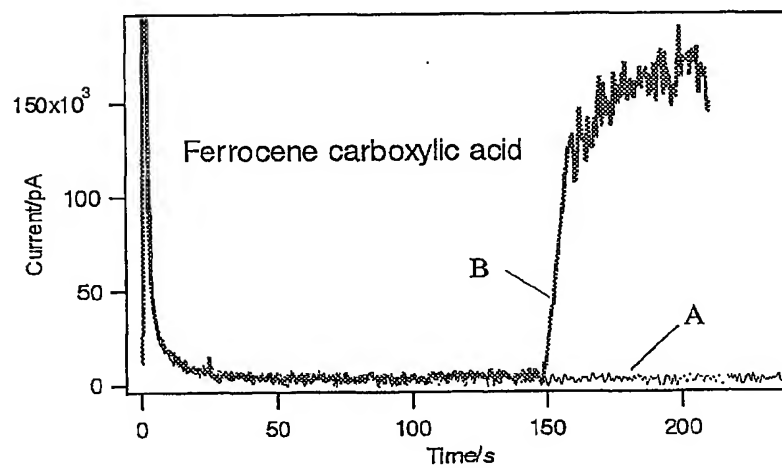


Fig. 14